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Mapping CD20 molecules on the lymphoma cell surface using atomic force microscopy

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Atomic force microscopy (AFM) was used to locate CD20 molecules on the surface of lymphoma Raji cells. Rituximab (a monoclonal antibody against CD20) molecules were linked onto the AFM tip via a polyethylene glycol (PEG) linker. Raji cells were adsorbed onto glass slides coated with poly-*L*-lysine. First, the CD20 distribution in a local area of the cell surface was visualized using the AFM lift scan mode. Second, 16 × 16 force curves were obtained from the same cell area to construct the CD20-rituximab binding force map. Finally, free rituximab was added to block the CD20 molecules on the cell surface and the lift phase image and CD20-rituximab force map were obtained again. The experimental results indicated that when the lift height was greater than the length of the PEG linker, no recognition sites were observed in the lift phase image. However, as the lift height decreased to the length of the PEG linker, some recognition sites were observed in the lift phase image and these sites were generally consistent with the pixels in the force map. After blocking, both the recognition sites in the lift phase image and the gray pixels in the binding force map decreased markedly. These results can improve our understanding of the distribution of protein molecules on the cell surface and facilitate further investigations into cellular functions.

atomic force microscopy, CD20, rituximab, lift scan, force curve

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Cells are the basic unit of living organisms. There are more than 100 trillion cells in the human body. These cells constitute the various tissues and organs, and have different morphologies and functions. In the human body, these cells do not work separately. They interact with their environment (other cells or the extracellular matrix) via their surfaces to regulate their own and other cells' behaviors [1]. On the cell surface, there are many different types of receptors that can recognize specific ligands in the extracellular environment. Cells use these receptors to communicate with their environment [2]. With the rapid development of cellular and molecular biology, it has become increasingly apparent that these receptors do not operate separately but are components of well-organized multimolecular assemblies on the cell surface [3]. Transient associations between the various molecules within microdomains on the cell surface can have a direct impact on the biological function of these molecules [4]. Therefore, understanding how these molecules localize, assemble and interact on the surface of living cells is important for elucidating the underlying mechanisms of cellular functions [4]. Several techniques can be used to observe the dynamic behaviors of individual molecules, including fluorescence microscopy, optical tweezers, electron microscopy, X-ray crystallography and nuclear

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magnetic resonance (NMR) [5,6]. For fluorescence microscopy, labeling may interfere with the molecular dynamics [5]; for optical tweezers, local heating can influence enzymatic activity and change the local viscosity of the medium [7]; while for the other techniques, the obtained structures are essentially static [6]. In recent years, atomic force microscopy (AFM) has attracted scientists' attention because of its outstanding capacity for observing the behaviors of single molecules under physiological conditions [8,9]. AFM achieves nanometer resolution and can work in various environments, including the liquids necessary for living cells. By tethering antibodies or ligands onto the AFM tip, specific molecules (receptors or antigens) on the cell surface can be located by using either binding force mapping [10] or dynamic force recognition mapping [11]. In this work, we combined the AFM lift scan method and the binding force mapping method to locate CD20 molecules on the surface of lymphoma Raji cells.

The principles of the AFM lift scan method [12] and the force mapping method [10] are shown in Figure 1. Rituximab molecules were linked onto the AFM tip. In the lift scan mode, the probe scans the surface twice. The first scan (called the main scan) is performed in the normal tapping mode to obtain the topography. Then, the tip is lifted to a constant height and the second scan (called the lift scan) is performed along the main scan trace. Therefore, the disturbance of the cellular topography is eliminated, and the lift scan trace is only influenced by the specific antibody–antigen binding between CD20 on the cell surface and rituximab on the tip. When rituximab binds to CD20, the amplitude of the cantilever changes and leads to changes in the phase information of the lift scan phase image, and hence CD20 on the cell surface can be visualized in the lift scan image. For force mapping, the local area of the cell surface is divided into an array of small areas (for example, 16 × 16), and then the functionalized probe is used to obtain a force curve for each small area. If CD20 molecules are present in the small area, then abrupt peaks may appear in the retraction portion of the force curve [13]. If CD20 molecules are not present in the small area, then the force curve is flat. The CD20-rituximab binding force for each small area is then computed from these force curves (if there is no abrupt peak in the force curve, then the binding force is indicated as 0). By normalizing these force values into the range of 0–255, the force map was then constructed using image processing software.

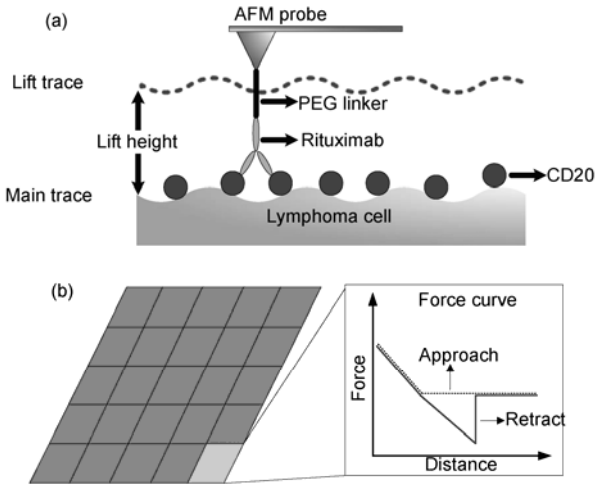

Figure 1 The principles of the AFM lift scan mode and force mapping. (a) Lift scan mode. The lift scan is performed along the main trace to eliminate the influence of cellular topography. (b) Force mapping method. An array of force curves is obtained to construct the binding force map.

Raji cells (a Burkitt's lymphoma cell line) and Hut cells (a T lymphoma cell line) were used in this study. The cells were cultured at 37°C (5% CO_2) in RPMI-1640 culture medium containing 10% fetal bovine serum for 24 h before experiments. Cells were harvested by centrifugation at 1000 r/min for 5 min. Then, the cells were dropped onto poly-*L*-lysine-coated glass slides and fixed with 4% paraformaldehyde. The PEG linker, NHS-PEG2000-MAL (JenKem Company, China), was used to link rituximab molecules onto the AFM tip. The functionalization procedure followed an established procedure [13] and RBITC-labeled goat anti-human IgG was used to verify whether rituximab molecules had been linked onto the AFM tip. In brief, the functionalized probe was placed into a Petri dish containing phosphate buffered saline (PBS), to which RBITC-labeled goat anti-human IgG was then added. After washing the unbound IgG, the probe was placed onto the stage of the fluorescence microscope (Nikon Ti-U-FL, Japan) to observe the fluorescence of the probe.

AFM imaging and measurements were performed using a Dimension 3100 AFM (Veeco Company, Santa Barbara, CA, USA) in PBS at room temperature. The spring constant of the cantilever (with a normal spring constant of 0.01 N/m) was calibrated using the thermal noise method. The functionalized probe was placed onto a Raji cell under the guidance of the AFM's optical microscope. First, the normal tapping mode was performed in a local area (3 μm) of the cell surface to obtain the cell topography. Then, the lift scan mode was performed at different lift heights (50 and 20 nm). Second, the 16 × 16 force curves were obtained from the same area to produce the CD20-rituximab binding force map. Then, free rituximab solution was added and about 30 min later, the normal tapping mode, lift scan mode, and force curves were obtained again. Also, the lift scan and force curve obtained with the functionalized probe were performed on Hut cells, which do not express CD20 molecules.

Figure 2 shows the results of mapping CD20 molecules on the Raji cell surface by combing the lift scan mode with binding force mapping. Figure 2(a) shows a fluorescence image of the functionalized probe. The functionalized probe exhibited bright fluorescence after staining with RBITC-labeled IgG, indicating that rituximab had been linked onto the AFM tip. In the optical image of the functionalized

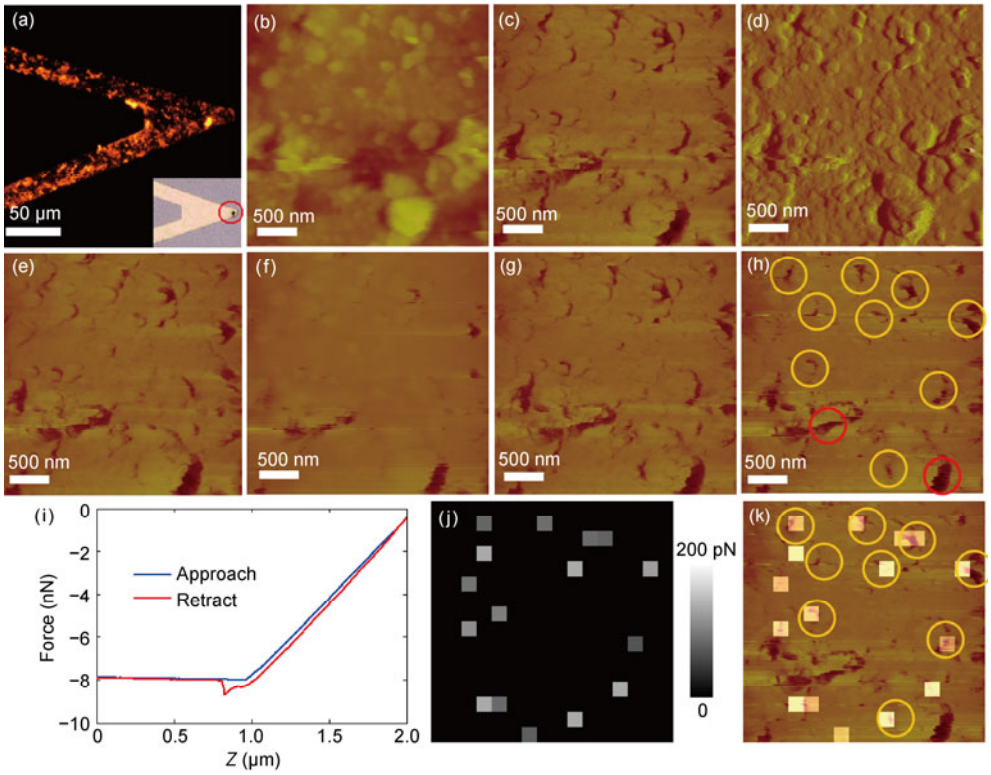

Figure 2 Analysis of the distribution of CD20 on the lymphoma cell surface. (a) Fluorescence image of the functionalized probe. Inset, optical image of the functionalized probe. (b)–(d) are AFM images acquired in the normal tapping scan mode. (b) Height image. (c) Phase image. (d) Amplitude image. (e)–(h) are AFM images acquired in the lift scan mode. (e) Phase image of the main scan with a lift height of 50 nm. (f) Phase image of the lift scan with a lift height of 50 nm. (g) Phase image of the main scan with a lift height of 20 nm. (h) Phase image of the lift scan with a lift height of 20 nm. (i) A typical force curve. (j) Force map. (k) Overlaying of the phase image (lift scan) and force map.

probe, the tip is clearly denoted by the circle (Figure 2(a)). During the functionalization procedure, both the cantilever and the probe tip were coated with antibodies [14] because both were made of silicon. Living cells have a soft and dynamic surface, which means that the scanning AFM tip can deform the cell surface [15]. Therefore, to avoid the deformation of the cell membrane, fixing agents are often used [11]. AFM images of the local cell surface obtained in the normal tapping mode revealed that the cell surface was rough (Figure 2(b)–(d)). Then, the lift scan mode was performed and the images obtained are shown in Figure 2(e)–(h). The phase images of the main scan (Figure 2(e) and (g)) were almost identical to the phase images obtained in normal tapping mode (Figure 2(c)). This is because the main scan during the lift scan mode is essentially the normal tapping mode. The molecular weight of the PEG linker used here is 2000 Daltons (information provided by the supplier). The length of a PEG linker with a molecular weight of 800 Daltons has been determined to be 8 nm [16,17]. Hence, the length of the PEG linker used here was about 20 nm. When the lift height was higher than the length of the PEG molecule, there was no recognition in the lift scan (Figure 2(f)). This is because the rituximab molecules could not contact the cell surface. When the lift height approximated the length of the PEG molecule, the recognition sites were seen in the lift scan, as denoted by the yellow circles in Figure 2(h). Some sites resembling the recognition sites were also seen, as denoted by the red circles in Figure 2(h). These sites were not recognition sites because they also appear in the phase image with a lift scan height of 50 nm. The cellular structures in these sites were quite high and may cause the changes in the phase signal.

Then, 16 × 16 force curves were obtained from the same area. Figure 2(i) shows a typical force curve with an abrupt peak in the retraction curve. From these 256 force curves, the CD20-rituximab binding forces were computed and the force map of the local cellular area was constructed, as shown in Figure 2(j). Overlaying the lift scan image and the force map shows that the two images were generally consistent with each other (Figure 2(k)). To verify the specific CD20-rituximab binding, two control experiments were performed. First, blocking experiments [18] were performed. After adding free rituximab, the distribution of the CD20 molecules was measured again, as shown in Figure 3(a)–(e). Very few spots were observed in the lift scan (Figure 3(c)). Furthermore, there were few recognition pixels in the force map, indicating that the distribution of CD20 molecules on the cell surface decreased significantly. This is because the added rituximab molecules bound to the CD20 molecules and hence masked these CD20 molecules. Then, the lift

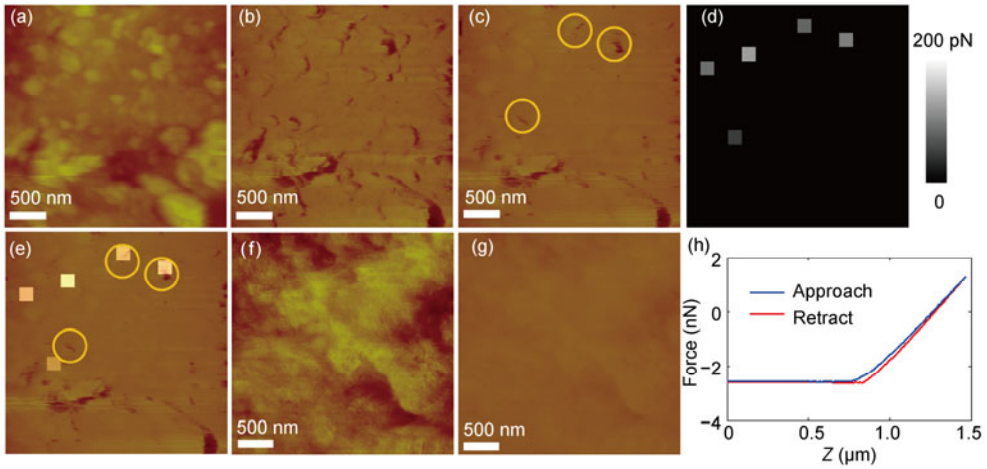

Figure 3 Control experiments. Detecting the distribution of CD20 molecules on the Raji cell surface after blocking (a)–(e). (a) Height image. (b) Phase image of the main scan with a lift height of 20 nm. (c) Phase image of the lift scan with a lift height of 20 nm. (d) Force map. (e) Overlaying of the phase image (lift scan) and force map. Performing the lift scan on Hut cells (f, g). (f) Phase image of the main scan with a lift height of 20 nm. (g) Phase image of the lift scan with a lift height of 20 nm. (h) A typical force curve obtained on Hut cells.

scan was performed on Hut cells. No recognition sites were seen in the phase image of the lift scan because Hut cells do not express CD20 molecules. Also, there were no abrupt peaks in the force curve (Figure 3(h)). Hence, the control experiments verified the specific molecular binding.

Since its invention, AFM has become a fascinating technology for investigating biological systems [19–22]. The rapid development of high-speed AFM in recent years has allowed researchers to watch single molecules in action in real time, directly providing biologists with unprecedented amounts of information on functional proteins instead of modeling dynamic processes [5,6,9]. When the systems being studied become more complex, AFM techniques that allow specific recognition of molecules will be vital, because many proteins do not show distinctive morphological features and therefore are indistinguishable from each other in an AFM image [5]. Here, we characterized the distribution of CD20 molecules on the lymphoma cell surface by combining the lift scan method with the binding force mapping method. However, there is a shortcoming of the lift scan method in that the cell surface is rough and corrugated, and this morphology may affect antigen-antibody binding. Therefore, the recognition image may not include all of the CD20 molecules in the cellular area local to the probe. Improving the detection probability of the functionalized AFM tip (particularly on living cells) is a challenge that needs to be addressed. In summary, CD20 molecules on the lymphoma cell surface were located by combing the lift scan method with the force mapping method. The experimental results validate the effectiveness of the lift scan method and facilitate further investigations into cellular functions.

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